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Degradation of Humoral Host Defense by *Candida albicans* Proteinase

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The effect of an extracellular proteinase from the pathogenic yeast *Candida albicans* on the bactericidal and opsonizing activities of human serum was studied. The ability of human polymorphonuclear leukocytes to kill *Staphylococcus aureus* was greatly reduced when the bacteria were opsonized with human serum treated with the proteinase. The reduction in the opsonizing activity of human serum was attributed to degradation of the Fc portion of immunoglobulin G by the action of *C. albicans* proteinase as determined by immunoprecipitation reaction. However, the Fab portion of immunoglobulin G was resistant to proteolysis by the proteinase. A clear reduction in the bactericidal activity of human serum against *Escherichia coli* was observed when the serum was treated with *C. albicans* proteinase. The reduction of serum bactericidal activity was attributed to the degradation of complement C3 by proteolysis by the proteinase as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, while C5 resisted the action of the proteinase. As determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the proteinase also degrades endogenous proteinase inhibitors, such as α_2 macroglobulin and α_1 proteinase inhibitor, which are involved in regulating inflammation. These results suggest that destruction of a host's defense-oriented or regulatory proteins facilitates debilitation of the infected host.

Systemic candidiasis and chronic mucocutaneous candidiasis are common infections in burn patients (20, 23) and in immunocompromised hosts (34) such as AIDS patients and organ transplant recipients (4, 16). An extracellular proteinase from *Candida albicans*, reported by Staib (33), Remold et al. (26), and Rüchel (28), has been characterized as a carboxyl proteinase (EC 3.4.23.6) has been considered an important virulence factor (2, 22, 25). The proteinase can break down a number of host substrates, including albumin (30), collagen (6, 7), immunoglobulin A (IgA) (28), keratin (21), and hemoglobin (26). To explain the pathogenic role of the proteinase in *Candida* infection, studies including immunochemical study of pathological sections of patients (31) and experimental infection with proteinase-deficient *Candida* mutants (11, 13) have been reported. The proteinase appears to facilitate adherence (1, 10), growth (35), and epithelium and tissue invasion (10). Although these observations suggest a potential role of the proteinase in virulence and many possible roles for the proteinase in pathogenicity have been proposed, very few studies have been carried out to determine the pathogenic mechanism of the proteinase.

We recently reported evidence that *Candida*, *Pseudomonas*, *Serratia*, and *Porphyromonas* proteinases and those from other microbes cause increased vascular permeability by activating one or more steps of the kallikrein-kinin system to release bradykinin, leading to inflammatory reactions (9, 14, 15, 19) and clinical symptoms thereof. Moreover, we found that the *Candida*, *Pseudomonas*, and *Serratia* proteinases were able to activate the blood clotting factors which allow initiation of blood coagulation (8).

In this study, to confirm the pathogenic role of the *Candida* proteinase, we tested the effect of the proteinase on the opsonic and bactericidal activities of human serum through a complement-mediated pathway by using a purified single-complement system and human serum in vitro.

MATERIALS AND METHODS

***C. albicans* and preparation of the proteinase.** We used *C. albicans* IFO strain 1385 (serotype A) maintained in 2% glucose–1% peptone–0.5% yeast extract medium (pH 5.6). A synthetic medium (yeast carbon base; Difco, Detroit, Mich.) supplemented with 0.2% bovine serum albumin (BSA) as a nitrogen source was used to induce production of the proteinase. The proteinase was obtained from a culture supernatant as described previously (9). The proteinase solution, in 2.1 mg of 5 mM citrate buffer (pH 6.7) per ml, was stored at −80°C before use.

Biochemicals. Human IgG Fc, human IgG Fab, anti-human IgG Fc antibody,

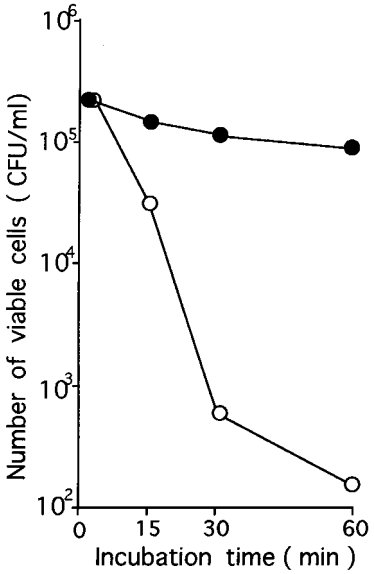

FIG. 1. Viable bacterial counts after incubation for 15, 30, and 60 min with PMNs. Bacteria were opsonized with untreated human normal serum (○) or that treated with *Candida* proteinase (●).

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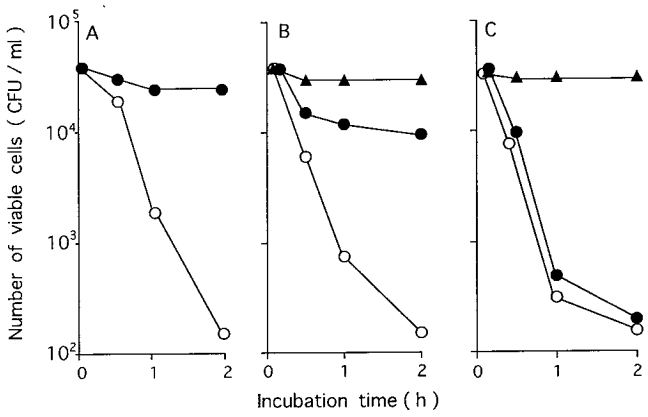

FIG. 2. (A) Viable bacterial counts after incubation for 0.5, 1, and 2 h with untreated normal human serum (○) or that treated with *C. albicans* proteinase (●). (B) Viable bacterial counts after incubation for 0.5, 1, and 2 h with C3-deficient serum alone (▲) or along with normal (○) or *C. albicans* proteinase-treated (●) C3. (C) Viable bacterial counts after incubation for 0.5, 1, and 2 h with C5-deficient serum alone (▲) or along with normal (○) or *C. albicans* proteinase-treated (●) C5.

and anti-human IgG Fab antibody were purchased from Organon Teknika N. V. Cappel Products. Human complement C3, human complement C5, human complement C3-deficient serum, and human complement C5-deficient serum were from Sigma Chemical Co., St. Louis, Mo. The media for cell culture were from GIBCO, Grand Island, N.Y. Human α_2 macroglobulin (α_2M) was from Calbiochem, La Jolla, Calif. Human α_1 proteinase inhibitor (α_1PI) was from Cosmo Bio Co., Ltd., Tokyo, Japan. Human plasmin and BSA were from Sigma. Unless noted otherwise, all other chemicals were purchased from Sigma. A synthetic substrate for plasmin, *t*-butyloxycarbonyl–Glu–Lys–Lys–4-methylcoumaryl-7-amide, was from the Peptide Institute, Inc., Osaka, Japan.

Preparation of serum and polymorphonuclear leukocytes (PMNs). Both whole and heparinized blood samples were obtained from a healthy volunteer. Whole blood was allowed to clot and was then centrifuged to separate the serum. Purified PMNs were prepared from heparinized samples by using a discontinuous Ficoll gradient system (5). Cell viability was estimated by trypan blue exclusion and was usually about 95%.

Killing of *Staphylococcus aureus* by PMNs in vitro. *S. aureus* 209P was grown overnight in nutrient broth (Difco) at 37°C, washed three times in saline, and resuspended and diluted in Hanks balanced salt solution. Human serum (100 μl) diluted 1:1 in 0.1 M acetate buffer (pH 4.0) was pretreated with the proteinase (15 μl, 2.1 μg/μl) for 1 h at 37°C. *S. aureus* was opsonized with proteinase-treated or nontreated serum at 37°C for 30 min. Purified PMNs (5×10^5) obtained from human peripheral blood were incubated with serum-opsonized *S. aureus* (2.5×10^5 CFU) in 1 ml of Eagle minimum essential medium at 37°C under gentle shaking (1.7 Hz). At 0, 15, 30, and 60 min, 50-μl samples were transferred into 5 ml of distilled water to destroy the PMNs, and viable counts of the bacteria were determined by using a 10-fold serial dilution and inoculating the dilutions on nutrient agar (Nissui, Tokyo, Japan) as usual.

Effect of *Candida* proteinase on the bactericidal activity of serum. *Escherichia coli* B was grown overnight in tryptic soy broth (Difco) at 37°C, washed three times in saline, and then diluted in Hanks balanced salt solution. Normal human serum (100 μl) was added to the *Candida* proteinase solution (30 μl, 2.1 μg/μl) and mixed with 945 μl of 50 mM acetate buffer at pH 4.0, which was then incubated at 37°C for 16 h. Tubes containing 5×10^4 *E. coli* cells and 100 μl of human serum treated with proteinase in 900 μl of Hanks balanced salt solution were incubated with gentle shaking (1.7 Hz) at 37°C for 2 h. Samples (100 μl) were withdrawn at 0, 0.5, 1, and 2 h, and survival of *E. coli* was determined by colony counting on nutrient agar plates. Non-proteinase-treated human serum controls were treated similarly.

Effect of *Candida* proteinase on the complement system. C3 and C5 were treated with the proteinase separately. First, 15 μg of protein of a complement C3 solution (15 μl) was treated with 3 μl of a proteinase (6.3 μg) solution in 32 μl of acetate buffer (50 mM, pH 4.0) containing 0.01% BSA, 0.15 mM $CaCl_2$, and 0.5 mM $MgCl_2$ for 6 h at 37°C. After treatment, the reaction mixture was added to 140 μl of Tris-HCl buffer (50 mM, pH 7.5) containing BSA and Ca^{2+}-Mg^{2+} as described above with 10 μl of C3-deficient serum (to supply the rest of the complement components other than C3) and 50 μl of *E. coli* (5×10^4 CFU/ml), and the mixture was incubated at 37°C for 0.5, 1, and 2 h. After incubation, 25-μl samples of the reaction mixture were diluted with Hanks balanced salt solution and withdrawn and the number of viable bacteria was determined by colony counting in nutrient agar pour plates. Heat-denatured (100°C, 5 min) proteinase was used as a control. C5 was also treated with the proteinase as described above.

Degradation of human serum components by *Candida* proteinase. Degradation of C3 and C5 by the proteinase was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by the method of Laemmli (12). Degradation of IgG fragments (Fc and Fab) by the proteinase was analyzed by the Ouchterlony immunoprecipitation reaction method. Degradation of α_2M and α_1PI by treatment with the proteinase was analyzed by SDS-PAGE. The effect of the plasmin-inhibitory activity of α_2M and α_1PI which were treated with the proteinase was determined by using the synthetic substrate *t*-butyloxycarbonyl–Glu–Lys–Lys–4-methylcoumaryl-7-amide as reported previously (17).

RESULTS

Effect of *Candida* proteinase on the opsonic activity of human serum. When *S. aureus* was opsonized with normal human serum and incubated with PMNs, more than 99% of the bacteria were quickly phagocytized and killed by the PMNs. In contrast, when *S. aureus* was opsonized with human serum treated with the *Candida* proteinase, at least 25% of the bacteria inoculated remained alive, even after 60 min of incubation (Fig. 1).

Effect of the *Candida* proteinase on bactericidal activity of serum. Figure 2A shows the bactericidal activity of normal human serum treated with *Candida* proteinase. When normal serum was used, a clear bactericidal effect against *E. coli* was observed. However, when proteinase-pretreated serum was used, the bactericidal effect was significantly reduced (Fig. 2A).

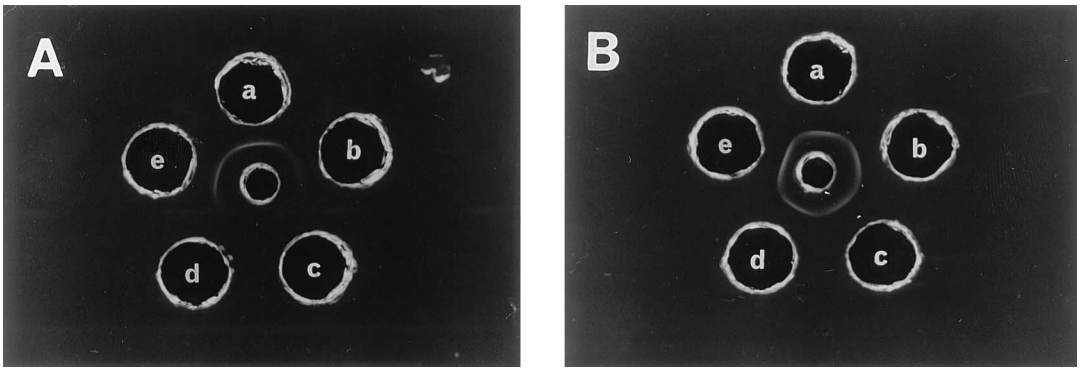

FIG. 3. Ouchterlony immunoprecipitation reaction of the purified Fc portion of IgG (A) and that of the Fab portion of IgG (B). (A) Wells: center, anti-Fc antibody; a, normal Fc; b to d, Fc treated with *C. albicans* proteinase for 3 (b), 6 (c), or 9 (d) min; e, Fc treated with heat-inactivated (100°C, 5 min) proteinase. (B) Wells: center, anti-Fab antibody; a, native Fab; b to d, Fab after treatment with *C. albicans* proteinase for 3 (b), 6 (c), or 9 (d) h; e, Fab after treatment with heat-inactivated (100°C, 5 min) proteinase for 9 h. These treatments of IgG Fc or Fab with *C. albicans* proteinase were carried out in 20 mM acetate buffer (pH 4.0) at 37°C; the molar enzyme/substrate ratio was 1:10.

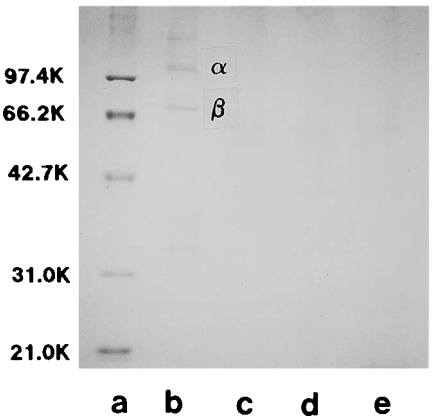

FIG. 4. Degradation of complement factor C3 by *C. albicans* proteinase as determined by SDS-PAGE. Lanes: a, molecular weight markers; b to 3, C3 as a substrate treated with *C. albicans* proteinase for 0 (b), 10 (c), 20 (d), or 30 (e) min. Conditions of proteinase treatment were as described in the legend to Fig. 3. α, α chain; β, β chain.

TABLE 1. Inactivation of human proteinase inhibitors by *C. albicans* proteinase[a]

Time of incubation with proteinase	% Inactivation of inhibitor	
	α_2M	α_1PI
0	0	0
30 s	ND	70
60 s	ND	100
0.5 h	45	ND
1 h	65	ND
2 h	100	ND

[a] Inactivation of α_2M (M_r, 800,000) and α_1PI (M_r, 50,000) by the proteinase was tested by determining plasmin inhibition activity. The enzyme and inhibitors at a molar ratio of 1:10 were incubated in 10 mM acetate buffer (pH 4.0) at 37°C. After different time intervals, a 50-μl aliquot was removed, a known concentration of human plasmin was added, and the mixture was incubated for another 10 min. Residual plasmin activity was measured by using the synthetic substrate *t*-butyloxycarbonyl–Glu–Lys–Lys–4-methylcoumaryl-7-amide and a fluorescence spectrophotometer with excitation at 380 nm and emission at 440 nm. Heat-denatured (100°C, 5 min) proteinase was used as a control. ND, not done.

To clarify the effect of the proteinase on the bactericidal action of the serum, we carried out purified-system assays with C3, C5, C3-deficient serum, and C5-deficient serum. As shown in Fig. 2B, C3-deficient serum showed no bactericidal action when it was used. In contrast, serum containing C3 showed significant bactericidal action. However, the bactericidal activity of the purified system diminished significantly when C3 was pretreated with the *Candida* proteinase. Likewise, the C5-deficient serum showed no bactericidal activity but serum containing C5 (normal) did exhibit bactericidal action. However, C5 treated with the proteinase showed virtually no change in bactericidal activity; its action was almost the same as that of control normal serum. This suggests that C5 was not affected by the proteinase (Fig. 2C).

Immunological analysis of degradation of serum components by the proteinase. Figures 3, 4, and 5 show the results of degradation of the Fc portion of IgG (IgG Fc and Fab), complement C3, and serum proteinase inhibitors α_2M and α_1PI caused by treatment with the proteinase. IgG Fc was hydrolyzed by the proteinase as shown by the results of the immunodiffusion precipitation assay. After 6 min of treatment with the proteinase, complete destruction of IgG Fc occurred and no trace of it was seen in the precipitin line (Fig. 3A). We also confirmed the degradation of IgG Fc by SDS-PAGE analysis (data not shown). However, IgG Fab was not affected by treatment with the proteinase; after 9 h of treatment, the fragment was still clearly observed (Fig. 3B). C3 was hydrolyzed by the proteinase and both the α chain (110 kDa) and the β chain (70 kDa) disappeared within 10 min after the start of incubation (Fig. 4). However, C5 resisted the proteolytic action of the proteinase (data not shown). The results of SDS-PAGE shown in Fig. 5 indicate that the proteinase degraded both α_2M and α_1PI markedly at 2 h and 60 s of treatment, respectively. The plasmin inhibition activity of α_2M and α_1PI was lost in a time-dependent fashion after treatment with the proteinase. (Table 1).

DISCUSSION

Effective participation of PMNs in the host defense depends on their ability to perform essential functions, i.e., adherence, chemotaxis, phagocytosis, and bactericidal activity (32). IgG and IgM are involved in the complement-dependent bactericidal mechanism, namely, opsonization and phagocytosis, in bacterial and fungal infections. Opsonization of target microorganisms with serum components is necessary for efficient phagocytosis. In the present study, we demonstrated that the proteinase from *C. albicans* was able to break down portions of the IgG and complement C3 molecules (Fig. 3 and 4), which are known to play a major role in humoral host defense mechanisms. The Fc portion of IgG molecules is responsible for

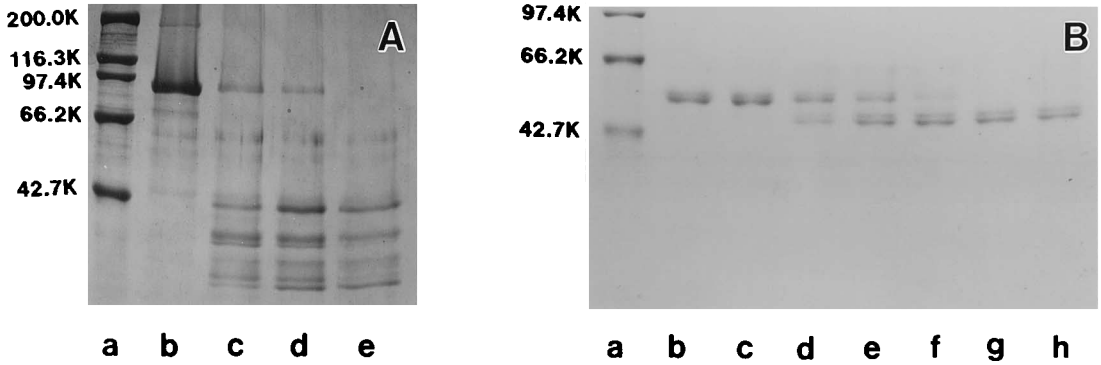

FIG. 5. Degradation of α_2M (A) and α_1PI (B) by *C. albicans* proteinase as determined by SDS-PAGE. (A) Lanes: a, molecular weight markers; b to e, α_2M as a substrate for treatment with *C. albicans* proteinase for 0 (b), 0.5 (c), 1 (d), or 2 (e) h. (B) Lanes: a, molecular weight markers; b to e, α_1PI as a substrate for treatment with *C. albicans* proteinase for 0 (b), 10 (c), 20 (d), 30 (e), 40 (f), 50 (g), or 60 (h) s. Conditions of proteinase treatment were as described in the legend to Fig. 3.

binding to the Fc receptor on the surface of phagocytes and for initiation of phagocytosis (3, 36). Degradation of the fragment resulted in a failure of phagocytosis and thus led to a decrease in the intracellular killing of bacteria by PMNs. This suggests that *C. albicans* proteinase inhibits the opsonic activity of IgG. It is also known that C3b and iC3b, derived from C3, act as opsonins (27). An obvious decrease in killing by PMNs was observed when human serum pretreated with the proteinase was used as the opsonin. Thus, *C. albicans* proteinase seems to reduce the opsonic function of immunoglobulin, as well as the bactericidal activity of complement derivatives C3b and iC3b, as demonstrated in Fig. 1 and 2.

It is well known that activation of the complement system by gram-negative bacteria can occur via either the classical or the alternative pathway (3). The classical pathway requires participation of the antigen-antibody complex to activate the cascade, whereas activation of the alternative pathway occurs by stimulation of C3 with lipopolysaccharide, zymosan, and other factors, even in the absence of antigen-antibody complexes. In the present study, an apparent decrease in bactericidal activity occurred in human serum treated with the *C. albicans* proteinase. To confirm this phenomenon, we carried out an experiment with purified C3, C5, C3-deficient serum, and C5-deficient serum. C3 treated with the *C. albicans* proteinase was not available for activation of the complement system via the alternative pathway to form a membrane attack complex. However, this proteinase had little effect on C5. This suggests that a strong bactericidal activity was preserved in the purified system composed of C5-deficient serum and proteinase-treated C5, as shown in Fig. 2C. It is suggested that the reduction of serum bactericidal activity could be attributed to degradation of these key factors by the proteinase.

α_2M and other endogenous proteinase inhibitors, such as α_1PI, C1 esterase inhibitor, antithrombin III, and α_2 antiplasmin are involved in critical regulation of various cascade systems, including the complement system, the kallikrein-kinin system, the fibrinolytic and clotting system, and inflammation reactions. We (9), Rüchel and Böning (29), and Neely and Holder (20) previously demonstrated that α_2M does not inhibit *C. albicans* proteinase activity; indeed, α_2M showed no inhibitory effect on the vascular permeability-enhancing activity of *C. albicans* proteinase through activation of the Hageman factor-kinin cascade. In the present study, SDS-PAGE showed decreases in the molecular weight of α_2M as a result of proteolytic cleavage by *C. albicans* proteinase. We previously showed that proteinases from *Serratia marcescens* degraded various serum proteins, including immunoglobulins and plasma proteinase inhibitors, and inactivated the complement system (17, 18). The role of these proteinase-producing, opportunistic pathogens in disease may be to degrade immunoglobulins and inactivate complements.

In conclusion, we determined that treatment of human serum with *C. albicans* proteinase affects the function of IgG Fc and C3, through which the *C. albicans* proteinase plays a role in augmenting pathogenesis. The breakdown of the humoral host defense mechanisms caused by the action of *C. albicans* proteinase may render hosts more vulnerable to microbial infections and aggravate infectious diseases in compromised hosts.

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